'Magic' scissors for genome surgery

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The efficient and permanent alteration of a human gene responsible for SCID provides a proof-of-principle demonstration of the potential of zinc-finger nucleases as human therapeutics.

A robust and reproducible means of specifically correcting 'faulty' bases in genes has been a longtime goal in genetic medicine. In a recent issue of *Nature*, Urnov *et al.*[1] use four-finger zinc finger chimeric endonucleases (ZFNs) to achieve highly efficient and permanent alteration of the gene encoding human interleukin 2 receptor γ (IL2Rγ), which underlies X-linked severe combined immune deficiency (SCID), commonly termed 'bubble boy disease.' The authors obtained a remarkable gene modification efficiency of 18% of treated cells without selection, 7% of which were altered on both X-chromosomes—a result that attests to the potential power of ZFN technology both as a research tool and in human therapeutics.

A long sought-after goal of molecular biologists has been the ability to manipulate or modify plant and mammalian genomes including the human genome at specific sites. Cells use the universal process of homologous recombination to mediate site-specific recombination and maintain their genomic integrity, particularly during the repair of a double-strand break (DSB), which otherwise would be lethal to cells. DSB repair of a damaged chromosome by homologous recombination, which works via the copy-and-paste mechanism, is the most accurate form of repair, using the homologous DNA segment from the undamaged chromosomal partner as a template. Gene targeting—the process of replacing a gene by homologous recombination—uses an extra-chromosomal fragment of donor DNA and invokes the cell's own repair machinery for gene conversion[2]. Gene targeting is not a very efficient process in mammalian cells—only about one in a million treated cells undergo the desired gene modification.

It has long been known that when a defined chromosomal break is introduced at a unique site within a genome, homologous recombination is induced at that site to repair the DSB in a large fraction of cells in a population[3]. The challenge has been to develop a general means of introducing a DSB at a unique chromosomal locus in the genome to induce homology-directed repair at that site with the exogenously added donor DNA.

ZFNs—proteins custom designed to cut at specific DNA sequences—then came to the rescue[4–6]. These artificial proteins combine endonuclease activity with the ability of zinc-finger domains to specifically recognize a base triplet in DNA. The Cys_2His_2 zinc finger motif can target specific sequences by virtue of its unique 30 amino acid structure (stabilized by a zinc ion), the α-helix inserting into the major groove of the double helix. Amino acids within the zinc-finger motif can be changed while maintaining the remaining amino acids as a consensus backbone to generate zinc-finger motifs with new triplet sequence specificities.

Normally, three such zinc-finger domains are linked together in tandem to generate a zinc finger protein that binds to a 9-bp site, which is a composite of the individual DNA triplet subsites recognized by each of the three zinc-finger motifs[7]. ZFNs thus combine the nonspecific cleavage endonuclease domain of *Fok*I restriction enzyme with zinc finger proteins to provide a general mechanism to introduce a site-specific DSB into the genome[4,5]. Binding of two three-finger ZFN monomers each recognizing a 9-bp inverted site (**Fig. 1**) is necessary because dimerization of the *Fok*I cleavage domain is required to produce a DSB[6,7]. Therefore, three-finger ZFNs effectively have an 18-bp recognition site, which is long enough to specify a unique address within mammalian genomes.

Reports from several laboratories using model systems have shown that designed three-finger ZFNs find and cleave their chromosomal targets in cells. As expected, they induce local homologous recombination at the site of cleavage[8–10].

In the *Nature* paper, Urnov *et al.* add an additional finger to the ZFN design because long-term overexpression of three-finger ZFNs was shown by others to be deleterious to human cells[9]. The authors posit that the additional zinc finger may confer increased specificity and selectivity to the ZFN. The resulting two four-finger ZFNs they create recognize and cut

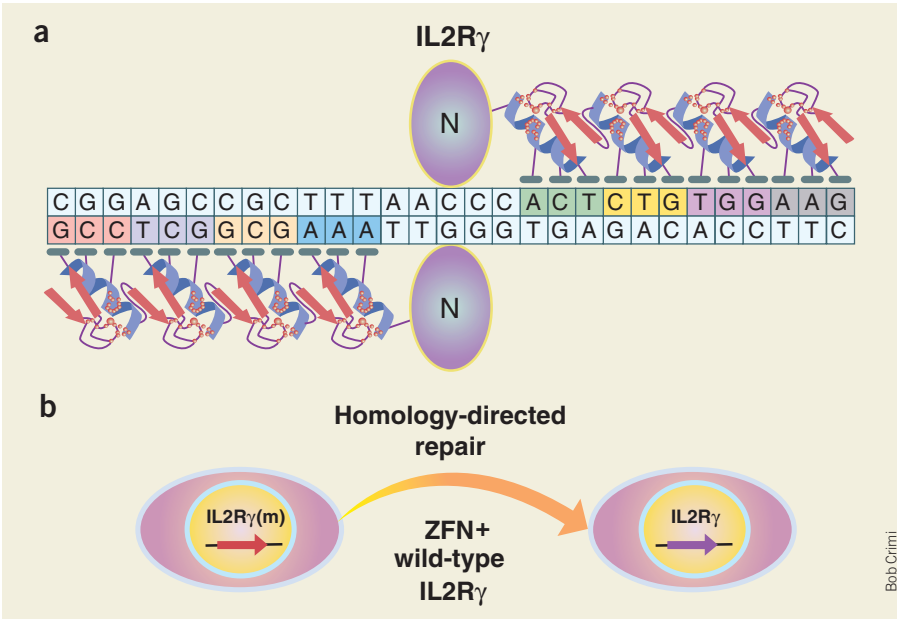

Figure 1 ZFN-mediated gene targeting in human cells. (**a**) Schematic representation of ZFNs bound to their targets within the IL2Rγ gene. (**b**) Gene editing of the SCID mutation by homology-directed repair in human cells. IL2Rγ(m) denotes a SCID mutation within the IL2Rγ gene. In this experiment, cells are cotransfected with both ZFNs and a fragment of the wild-type IL2Rγ gene.

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a 24-bp site (**Fig. 1**) in the gene encoding IL2Rγ. The authors optimize these ZFNs for sequence-specific cleavage by tinkering with individual zinc-finger motifs in the zinc finger protein and then test the ability of the altered ZFNs to mediate correction of a mutated green fluorescent protein (GFP) gene.

ZFN optimization in HEK293 cells is achieved by monitoring gene correction frequency of a single copy of a chromosomal GFP reporter gene, which is disabled by the insertion of a fragment of IL2Rγ gene containing the ZFN recognition sites. Several days after transient co-transfection of these GFP(–) cells with ZFN and GFP donor plasmid, FACS is used to quantify the GFP(+) cells and thereby identify the optimal ZFN. The GFP gene encoded in the donor plasmid has its first twelve base pairs and the start codon deleted to prevent its expression in cells. The donor plasmid used for *in vivo* gene editing contains a fragment of the IL2Rγ locus, which is altered to carry a silent point mutation (overlaps the codon for proline at position 229) to create a novel *Bsr*BI restriction enzyme site in exon 5. By using the optimized ZFN and the donor plasmid, Urnov *et al.* achieve highly efficient and permanent modification of the sequence at the endogenous IL2Rγ locus. Thus, the sequence at the IL2Rγ locus in human cells is altered from 5'-CCA CTC-3' to 5'-CCG CTC-3' by recombination with the donor plasmid. The *Bsr*BI restriction site also overlaps the SCID missense mutation site at T703C (Leu230Pro).

Furthermore, Urnov *et al.* use ZFN-mediated HR to alter or correct the endogenous expression of IL2Rγ gene in K562 cells. In a first step, they introduce a single base-pair frameshift concomitant with a DraI recognition site in exon 5 and alter IL2RÁ gene expression. In a second step, they restore IL2Rγ gene expression in the mutant cells by ZFN-mediated gene editing using the donor plasmid containing the *Bsr*BI restriction site. The ZFN-driven targeted alterations are confirmed by quantifying mRNA and protein levels in these cells.

Although the Urnov *et al.* paper is an important proof of principle, several challenges face ZFN-mediated gene targeting before its full potential can be realized. First, for each desired gene correction, a lengthy design or selection approach must be employed to identify the correct zinc finger protein to bind the target sequence for use in the ZFN. Although selection approaches yield the desired high-affinity ZFP, they are very tedious and cumbersome to perform.

For the present, therefore, design remains the method of choice[6]. This approach requires the identification of a target site in the gene of interest near the mutation site and creation of the ZFN by a simple oligonucleotide assembly strategy using PCR. The zinc finger designs for all GNN and ANN triplets are now available in the literature for assembling the zinc-finger proteins. Although some of the zinc-finger designs for TNN and CNN triplets are known, the complete set of zinc finger designs is not yet published; it remains in the proprietary database of Sangamo BioSciences (Richmond, CA, USA)—the company that sponsored the Urnov *et al.* study.

Second, because DSBs are lethal, an alternative pathway also exists in cells called nonhomologous end joining, which competes with homology-directed repair of the DSB. Repair by nonhomologous end joining is achieved by simple ligation; it is by nature mutagenic. Therefore, it is crucial to establish that unwanted alterations do not occur at nonhomologous sites in other parts of the genome through nonhomologous end joining because of residual cleavage by the ZFNs as a result of their binding either at secondary degenerate sites or at single ZFN binding sites[6]. The genomic integrity and stability of treated cells needs to be intact at all other loci, except at the targeted chromosomal site after the ZFN treatment.

Third, continued overexpression of the three-finger ZFNs was shown to be harmful to human embryonic kidney 293 cells. Increasing the sequence specificity and selectivity by adding more fingers to ZFN appears to solve this problem. Methods for regulated or transient expression of the ZFNs within cells would further reduce cytotoxicity[6].

Fourth, ZFNs to more genes need to be designed and tested in different cell types, including primary cells and stem cells, which may be more sensitive to apoptosis signals. This will confirm that homology-directed repair is a universal process and that ZFN-mediated gene targeting is broadly applicable in all cell types.

And finally, although it appears that both alleles of a gene within cells can be modified using this approach, it may not be a very robust process. Multiple rounds of treatment with ZFNs may be required.

As these problems are addressed, ZFN-mediated gene targeting is likely to become a powerful research tool in the repertoire of molecular biologists for 'gene editing' and 'directed mutagenesis' of plant and mammalian genomes. As the Urnov *et al.* paper indicates, ZFNs also show great potential as therapeutics in the treatment of monogenic disease (see **Box 1**), although the technology clearly remains in its early stages of development.

Box 1 ZFN-mediated gene targeting and gene therapy

Gene therapy provides a new paradigm for treating genetic disease by correcting the causative gene defect. Current gene therapy protocols, however, use gene addition approaches to compensate for the faulty gene. Therapeutic genes are delivered and inserted randomly within the genome using viral vectors. Such random insertions of the therapeutic gene are by nature mutagenic and can give rise to cancer phenotypes by the activation of oncogenes as has been observed with gene therapy trials to treat SCID disease[11]. Since ZFN-mediated gene targeting uses the cell's own homology-directed gene editing process rather than gene addition, the hope here is that many of the difficulties associated with current gene therapy protocols will be circumvented.

The first applications of ZFN-mediated gene targeting, as a form of gene therapy to treat human disease, will likely occur in *ex-vivo* gene therapy using stem cells. Here, the gene-modified autologous cells can be identified, expanded in culture and replenished into the patients. Currently, several laboratories are working to create targeted Δ32 deletion at the chromosomal loci encoding the CC chemokine receptor type 5 (CCR5) gene in hematopoietic stem cells ($CD34^+$ cells) of individuals who are at high risk for HIV infection[6]. CCR5 is the major coreceptor on $CD4^+$ cells, through which HIV gains entry into cells. The virus is unable to infect $CD4^+$ lymphocytes and macrophages that are homozygous for the CCR5(Δ32) allele. The individuals homozygous for the CCR5(Δ32) allele (who are otherwise healthy) lack functional CCR5 expression and are highly protected against HIV-1 infection. Other laboratories are working to tackle sickle-cell anemia as well.

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